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Protection by live *Mycobacterium habana* vaccine against *Mycobacterium tuberculosis* H37Rv challenge in mice

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Background & objectives: **In recent years the efficacy of BCG vaccine against tuberculosis has been questioned and there is no alternative vaccine available. Several strategies are being applied to get a satisfactory vaccine. Two approaches are generally considered: the subunit vaccines and the whole cell vaccines. The objective of this investigation was to evaluate an avirulent mycobacteria,** *Mycobacterium habana***, as a whole cell vaccine to protect mice from infection of** *M. tuberculosis* **H37Rv.**

Methods: **AKR and immunocompromised SJL/J mice were immunized with live** *M. habana* **vaccine. These mice were challenged with** *M. tuberculosis* **H37Rv eight weeks later along with unimmunized control mice. Protection by** *M. habana* **vaccine was measured through several parameters, which included survival of challenged mice, dissemination of challenge strain and histopathology of lung tissues.**

Results: *M. habana* **vaccinated animals were healthier than the unvaccinated mice after challenge with** *M. tuberculosis* **and survived with significant increase in mean survival time. The viable count of challenge strain was at least 100-fold less in vaccinated mice than the control mice. The lung tissues in unvaccinated mice showed marked bronchopneumonia with clusters of acid fast bacilli, whereas vaccinated mice showed small areas of damage and evidence of protection subsequently.**

Interpretation & conclusion: **It may be concluded from the evidence presented here that mice vaccinated with** *M. habana* **were protected from challenge with** *M. tuberculosis* **in both normal and immunocompromised states.**

Key words *Mycobacterium habana* vaccine - *M. tuberculosis* challenge - protection - tuberculosis

Tuberculosis has become a major health problem of the world[1], which would be controlled by a judicious combination of chemotherapy and vaccination. Although vaccination would be cost effective, suitable vaccines are not available. The efficacy of BCG has recently been doubted[2]. BCG has proven to be efficacious in preventing systemic childhood TB including TB meningitis but not pulmonary TB in adults[3]. Therefore, there is a need for a vaccine which would be effective against sensitive as well as multiple drug resistant bacilli, and be a safer vaccine that would not reactivate in immunocompromised individuals. Two strategies are being pursued in vaccine development, the subunit approach in which immunity is induced by unique antigens and the whole cell approach relying on multiple antigens[4-6]. In the latter approach, auxotrophic *Mycobacterium tuberculosis* with attenuated virulence, auxotrophic and recombinant BCG and avirulent mycobacteria have been studied[7-11]. In this chain of avirulent mycobacteria as a vaccine against TB, we have evaluated live

M. habana vaccine to protect mice against challenge with *M. tuberculosis.*

Material & Methods

Mice: Inbred females, weighing 18-20 g, AKR and SJL/J mice, obtained respectively from, Rockefeller University, USA and Jackson Laboratories, USA, were bred in this Institute and maintained on Hindustan pellet diet and water *ad libitum.* SJL/J mice are immunocompromised having defective T cell receptor induced IL-4 production and absence of T cells with the NK 1.1 antigen.

Mycobacteria, culture, vaccination and infection: M. habana TMC 5135 and *M. tuberculosis* H37Rv TMC102 were procured from Trudeau Mycobacterial Culture Collection Centre, N.Y., and maintained on Lowenstein - Jensen (L-J) medium[12]. For viable counts (cfu), Middlebrook 7H10 agar supplemented with oleic acid, albumin, dextrose and catalase (OADC) was used[13]. Culture media and constituents were purchased from Beckton and Dickinson, USA. Culture was harvested from L-J slants and appropriate suspension was made in normal saline (0.85%) with Tween-80 (0.05%). Mice (AKR and SJL/J) were injected iv via the lateral vein with 10^6 cfu of live *M. habana* in 0.2 ml volume. After 8 wk, vaccinated and unvaccinated groups of mice were challenged with 10^6 cfu of *M. tuberculosis* H37Rv by iv route. Mice were sacrificed at desired periods and bacterial counts were studied in the lungs, spleen and liver and histopathological examination of the lung was done. For bacterial counts, the organs were homogenized separately in sterile normal saline with Tween-80 and serial dilutions were plated and incubated at 37°C till visible colonies appeared. Protection to challenge with *M. tuberculosis* H37Rv was assessed by comparing several parameters between unimmunized and *M. habana* immunized mice. Ten mice were taken in each group and three mice were sacrificed for viable counts in various tissues on day 10, 60 and 90 post vaccination. The parameters included weight of the mice and visceral organs, necropsy score, dissemination of challenge strain in the lung, liver and splenic tissues, histology of lung, survival and mean survival time (MST) of mice. The lesions in the visceral organs were given necropsy scores depending on the number and intensity, from 0 to 4 as described earlier[14]. Few miliary nodules were given a score of 1 and patchy coalesced intense lesions were given a score of 4, whereas intermediary lesions were given scores of 2 and 3. MST for a defined period of time (30 days) was determined as the sum of the individual animals which survived expressed in days divided by the total number of animals. Infection of animals, vaccination and sacrifice were carried out in the experimental wing of the animal house. The processing of the organs for viable counts and histopathology was carried out in the biosafety cabinet. Disposal of animals and organs was carefully executed in the incinerator. This study was approved by the Animal Ethics Committee of the Institute.

Histology: Lung tissues of mice from different experimental groups were trimmed in pieces of 2-3 mm thickness and dehydrated with three changes of acetone (15 min each). Tissues were then kept in 1:1 mixture of acetone and benzene for 15 min and cleared in benzene with three changes of 20 min each. Tissues were then impregnated with paraffin wax with three changes of 3 h each. Paraffin sections were cut on a rotary microtome, and stained for acid fast bacilli[15]. Briefly, deparaffinisation was done in xylene-peanut oil mixture (2:1), two changes of 12 min each at room temperature. Ziehl - Neelsen staining with Carbol fuchsin (Sigma, USA) was done at room temperature for 30 min. Excess stain was washed out with water and the sections were decolourized with 5 per cent H_2SO_4 in 25 per cent ethyl alcohol until light pink in colour. Sections were then washed with water for 5 min and counterstained with Harris haematoxylin (Hi Media, Mumbai) for 30 sec. Sections were then washed again with water, dehydrated with acetone and put into xylene. Finally they were mounted with DPX and light microscopic examination of paraffin sections of tissues was done after staining them with haematoxylin and eosin (Sigma, USA) using standard staining methods[16].

Statistical analysis: The log mean of the parameters were compared by Student's t-test.

Results & Discussion

To find out whether vaccination of mice with live *M.habana* was safe, dissemination of live *M. habana* in various organs of vaccinated mice was monitored. Significant reduction ($P<0.05$) in the counts of *M. habana* was observed in the tissues 60 days post vaccination (Table I). The load of bacilli was found 10-fold more in liver and spleen compared to lung. There was significant clearance of *M. habana* from the lungs. The appearance of visceral organs was the same as in unvaccinated mice. There was no evidence of lesions in the lung tissues 60 days post vaccination with *M. habana*. After 90 days nearly complete clearance of *M. habana* was observed from all tissues as evident from tissue smear staining for the bacilli and only few colonies growing on the plates. None of the vaccinated mice died, indicating that live *M. habana* immunization was safe in mice.

The effect of *M. habana* vaccination on protection against challenge with *M. tuberculosis* was evaluated. The unvaccinated AKR and immunocompromised SJL/J mice appeared sick and sluggish in disposition with ruffled hair after 8-10 days of challenge whereas those in the vaccinated groups were healthier and active. A decrease of 2 g in average weekly body weight was observed in the unvaccinated group of mice after challenge. The average necropsy scores and weight of the visceral organs in unvaccinated and *M. habana* vaccinated mice are shown in Table II. As compared to the vaccinated mice, the average weight of lung and necrotic lesions were about 30 and 50 per cent more in the unvaccinated mice after *M. tuberculosis* challenge. Similarly necropsy score was 60-70 per cent more in unvaccinated mice compared to the vaccinated mice and the liver in the former group of mice was enlarged. The *M. habana* vaccinated mice were healthier than unvaccinated mice after challenge with *M. tuberculosis*.

Dissemination of *M. tuberculosis* was followed in the lung, spleen and liver of vaccinated and unvaccinated mice. The cfu found in these organs was at least 100-fold less in immunized mice as compared to the unvaccinated control (Table III). It is significant that the effect of the vaccine in restricting the multiplication of *M. tuberculosis* was evident in immunocompromised SJL/J mice as in the normal AKR mice, although the proliferation of challenge bacilli was about one order of magnitude higher in SJL/J mice (Table IV).

Table I. Viable counts of *M. habana* in various tissues of AKR mice

Time	Lung		Spleen		Liver	
	cfu/g	Mean $\log_{10}$	cfu/g	Mean $\log_{10}$	cfu/g	Mean $\log_{10}$
Day 10	2.5×10^4	4.39±0.67*	1.6×10^5	5.20±0.60*	1.1×10^5	5.04±0.80*
Day 60	1.0×10^2	2.60±0.37	2.0×10^3	3.30±0.40	1.0×10^3	3.00±0.41

Ten AKR mice were vaccinated with 10^6 cfu of *M. habana*. Three mice were sacrificed on 10, 60 and 90 days post-vaccination for viable counts. On day 90 very few colonies were observed on the plate. *$P<0.05$ compared to day 60

Table II. Necropsy score and weight of visceral organs of vaccinated and control AKR mice after challenge of *M. tuberculosis*

Group	Lung		Spleen		Liver	
	score	weight	score	weight	score	weight
Vaccinated	1.2±0.06*	2.77±0.12*	0.77±0.04*	3.32±0.3	0.8±0.04*	2.58±0.21*
Unvaccinated	3.2±0.2	3.81±0.1	2.25±0.13	3.50±0.16	2.21±0.22	3.92±0.34

Ten AKR mice were taken in vaccinated and control groups. Mice in the vaccinated group were immunized with 10^6 cfu of *M. habana*. After 8 wk, both groups of animals were challenged with 10^6 cfu of *M. tuberculosis* H37Rv. Observations on necropsy score and weight were made in three weeks after challenge. *$P<0.05$ compared to unvaccinated group

When expressed in terms of death caused by *M. tuberculosis* infection, it was observed that the unvaccinated mice died within three to four weeks of challenge. Vaccination was clearly found to enhance the survival and protected the mice (Fig. 1) and the mean survival time was enhanced by 50 per cent, indicating that *M. habana* vaccine definitely protected the mice from death caused by *M. tuberculosis* infection.

Histological examination of the lung tissues in unimmunized but *M. tuberculosis* challenged mice showed marked acute bronchopneumonia. Granulomatous lesions appeared with the passage of time. There were fairly large number of acid fast bacilli in clusters and macrophages were seen laden with bacilli. The lung lesions tended to be moderately large with scattered infiltrates of lymphocytes admixed with epithelioid cells (Fig. 2 A, B).

By contrast, AKR mice immunized with *M. habana* showed evidence of protection after 2 months of challenge with *M. tuberculosis.* The protective effect became more established after 3 months. Areas of damage in the lungs of vaccinated

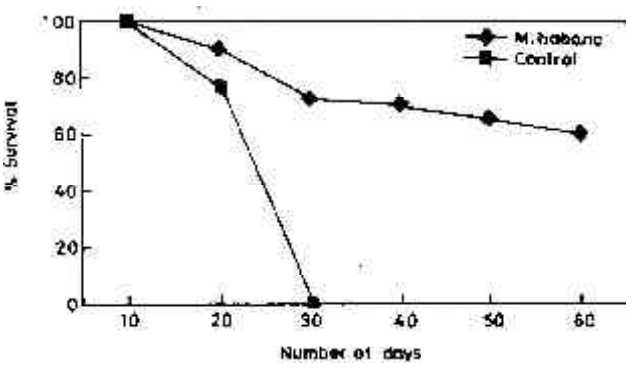

Fig. 1. Per cent survival of unvaccinated AKR mice and corresponding *M. habana* vaccinated mice after challenge with *M. tuberculosis.*

mice were limited to a small portion of the pulmonary parenchyma. The lesions were small and compact with respect to the surrounding parenchyma and had increased number of lymphocytes throughout the lesion. No necrosis was observed in the vaccinated group (Fig. 2 C, D). The findings in the lung tissues of unimmunized and vaccinated SJL/J mice were very similar to those in normal AKR mice. The unvaccinated group challenged with *M. tuberculosis* showed relatively larger areas of

Table III. Viable counts of *M. tuberculosis* in various tissues of AKR mice

Group	Lung		Spleen		Liver	
	cfu/g	Mean $\log_{10}$	cfu/g	Mean $\log_{10}$	cfu/g	Mean $\log_{10}$
Vaccinated	1.42×10^5	5.00±0.41**	5.8×10^4	4.72±0.28	4.3×10^5	5.31±0.40*
Unvaccinated	1.70×10^7	7.20±0.67	6.2×10^5	5.81±0.84	6.2×10^6	6.84±0.72

Ten AKR mice were taken in vaccinated and control groups. The protocol mentioned in Table II was followed. Viable counts were made in three weeks after challenge.

*P<0.05, **P<0.01 compared to unvaccinated group

Table IV. Viable counts of *M. tuberculosis* in various tissues of SJL/J mice

Group	Lung		Spleen		Liver	
	cfu/g	Mean $\log_{10}$	cfu/g	Mean $\log_{10}$	cfu/g	Mean $\log_{10}$
Vaccinated	2.0×10^6	6.30±0.04*	1.6×10^5	5.20±0.30*	1.8×10^6	6.25±0.42*
Unvaccinated	3.2×10^8	8.50±0.74	8.1×10^6	6.90±0.60	4.6×10^7	7.66±0.33

Ten SJL/J mice in each group were taken. The protocol mentioned in Table II was followed. Viable counts were made in three weeks after challenge.

*P<0.05 compared to unvaccinated group

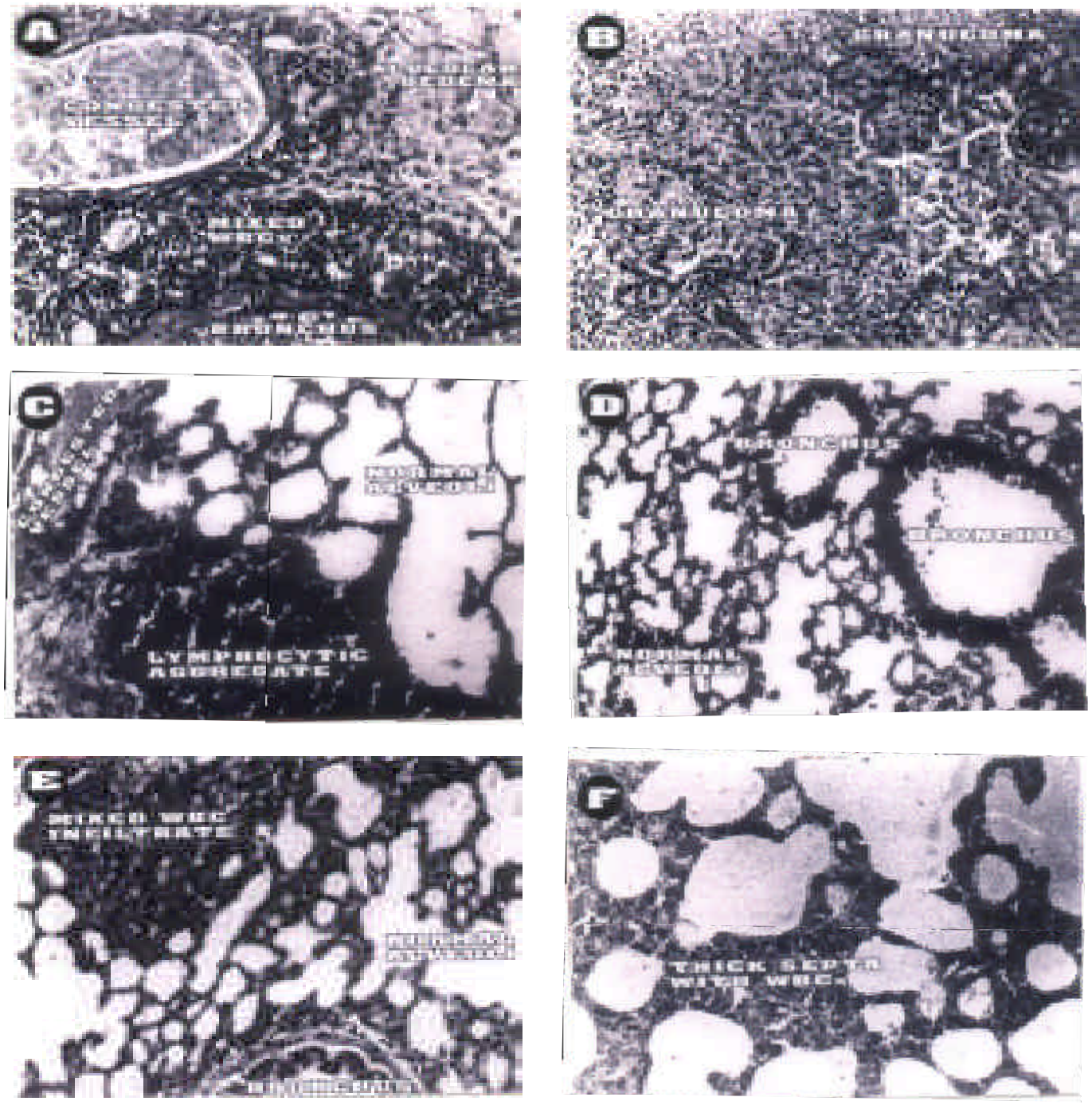

Fig. 2. Comparative histology of lung parenchyma of unvaccinated and vaccinated mice after challenge with *M. tuberculosis* H37Rv. A, B: unvaccinated AKR mice presented with extensive peribroncheal and alveolar leucocytic infiltration, areas of necrosis, oedema and vascular congestion (A); formation of granulomas was apparent in later stages (B); C, D: *M. habana* vaccinated AKR mice presented with clearing of alveolar oedema and necrosis, leucocytic infiltrates consisted mainly of lymphocytes (C); near complete clearance of inflammatory exudates and restoration of normal lung histology in later stages (D); E, F: *M. habana* vaccinated SJL/J mice also presented with marked diminution of inflammatory exudates (E); however some degree of septal thickening and mixed leucocyte (WBC) infiltrates consisting mainly of lymphocytes and monocytes could be detected even in the later stages (H & E; A, B, F: X 200; C, D, E: X 100).

damage in the lung parenchyma, along with areas of necrosis and unorganized lymphocyte infiltration whereas the vaccinated group developed lung lesions that were small and of limited extent, lacked pulmonary necrosis and granuloma formation, and had a fairly high percentage of lymphocytes within the lesions (Fig. 2 E, F).

The BCG vaccination against TB has been in use in most parts of the world but doubts have been raised about its effectiveness. The result of the south India trial of BCG vaccine[2] underlined the need to have an improved vaccine against tuberculosis. There is a need to look beyond BCG for a more effective TB vaccine. Various attempts have been made in the recent years. The purified antigens of *M. tuberculosis* have been evaluated in experimental models but they did not induce sustained immunity[17], probably as they needed to be delivered in adjuvant. Contrary to the use of subunit vaccine, the whole cell vaccines would not require the use of adjuvant. With this rationale leucine auxotrophs of *M. tuberculosis* were tested with the hope that such vaccines would be attenuated by their inability to grow, however the inability of auxotrophic mutants to grow failed to stimulate memory T-cells[7]. The danger of reversion of auxotrophs to prototrophs should also be seriously considered because reverted vaccine strains are expected to be fully virulent. The safety of mutant vaccine strains of *M. tuberculosis* needs to be fully ensured in HIV infected immunocompromised subjects. The use of avirulent and antigenically relevant mycobacteria as vaccine against *M. tuberculosis* infection offers several advantages and should be explored thoroughly. Such vaccines are not likely to present vaccinees with the risk of TB in immunocompromised state, whereas BCG, which is derived from *M. bovis*, always carries the threat. *M. habana* vaccine, used in this study, appears to be a potential protective immunogen. The immunological responses induced by *M. habana* should be thoroughly investigated. It has been shown that many peripheral and integral membrane proteins of *M. habana* are same as that of *M. tuberculosis*[18-20]. Therefore *M. habana* appears to be a good choice for further studies as candidate vaccine for tuberculosis.

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